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FOREWORD

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J. Don Chen 9/29/98
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(5) Introduction

All-trans retinoic acid (RA) is known to inhibit growth of estrogen receptor (ER)-positive human breast carcinoma (HBC) cells (25, 26, 31). The action of RA is mediated by its intracellular receptor known as retinoic acid receptors (RARs) that belong to the steroid/thyroid hormone receptor superfamily (20). These receptors are DNA-binding proteins and their activities are regulated by hormones. It is known that 17 β -estradiol (E2) can promote the growth of ER-positive HBC cells, while 4-hydroxytamoxifen (HTM) acts as an antiestrogen which may potentiate RA-induced growth arrest of HBC cells (2, 3, 9, 10, 30). The use of RA or its synthetic analogs as therapeutic agents in treating breast cancer is promising. However, how RA inhibits the growth of HBC cells and how it interacts with E2 are currently unclear. In this project, we plan to investigate the mechanisms of the inhibitory effects of RA on HBC cells and the cross talk between RA and E2.

To understand how RA inhibits the proliferation of ER-positive HBC cells, several laboratories have studied the expression and regulation of the RAR genes in different HBC cell lines. It was found that RAR α is expressed at a higher level in ER-positive cells than in ER-negative cells, while RAR β is expressed in some ER-negative cells and RAR γ in both ER-positive and ER-negative cells (23). Furthermore, RAR α is up regulated by estrogen (22) which explains the high level expression of RAR α in ER-positive HBC cells. By using retroviral vector-mediated gene transduction to introduce the RAR genes into RAR-negative HBC cells, it was shown that three isoforms of RARs can mediate RA-induced growth arrest and apoptosis in HBC cells (11, 26, 28). RAR can repress target gene expression in the absence of hormone. RA acts as a switch to release the repression and to stimulate transcriptional activation. It is possible that the mechanisms of RA-mediated growth arrest in HBC cells is due to the induction of gene products which may block cell proliferation. The induction of apolipoprotein D gene by RA has been shown to correlate with growth arrest and cell differentiation in HBC cells (19), however, the precise mechanisms of this RAR-mediated growth inhibition of HBC cells remains unknown.

Recently, several nuclear receptor associated proteins have been reported (4, 14-18, 21, 29). These proteins function as cofactors that help the receptors to activate or to repress target gene expression. The nuclear receptor corepressor SMRT (silencing mediator for retinoid and thyroid hormone action) functions as a transcriptional corepressor that can promote repression activity of unliganded RAR (4). The unliganded RAR possess strong transcriptional repression activities and natural RAR mutants exhibit activities that cause oncogenic transformation and/or blockade of cell differentiation (7, 8, 12, 13, 27). The biological effects of these mutants suggest that regulation of transcriptional repression activity is an important aspect in hormone action. It is believed that the release of repression is due to dissociation of corepressors. Since RAR plays an important role in the regulation of HBC cell proliferation, regulation of RAR activity by its cofactor should be important as well in regulating breast cancer development. Because transcriptional repression is involved in the oncogenic transformation induced by nuclear receptors, we propose to investigate the involvement of nuclear receptor corepressors in the oncogenic process of HBC cells. We have thought of a hypothesis that suggest how SMRT may

be involved in breast cancers and how this cofactor may mediate cross-talk between estrogen and RA in regulation of HBC cell growth and proliferation.

Our hypothesis:

We propose a hypothesis that integrates the role of nuclear receptor corepressors in the signal transduction pathways of RA and E2 (see Figure 1 in Appendices). As suggested by this model, the unliganded RAR will associate with corepressors in HBC cells. Such association is required transcriptional repression by RAR. Repression of RAR target genes will then inhibit HBC cell differentiation. If this model is correct, the level of SMRT protein will be critical for regulation of HBC cell differentiation. RA induces dissociation of RAR and the corepressors and stimulates the transcriptional activation by recruiting coactivators. Thus, simultaneous anti-repression and activation by RA will rapidly induce target gene expression to trigger cell differentiation and/or apoptosis.

Activation of ER by E2 enhances expression of ER-target genes, which encode new protein to stimulate breast cancer cell proliferation. On the contrary, antiestrogen promotes association between ER and corepressors. We have shown that SMRT interacts with ER weakly and binding of E2 could not release SMRT from ER. This result implies a distinct function of SMRT in regulation of ER function. Since hormone and anti-hormone may induce distinct conformations, we have proposed that anti-hormone will induce a conformation that permits stronger association with SMRT. This model could explain action of E2 antagonist and suggest an integrator role of SMRT between RA and E2 signaling.

This model can also explain the inhibitory effect of RA on ER-positive breast cancer cells. Upon treatment of breast cancer cells with retinoic acid, SMRT dissociates from the ligand-bound RAR, resulting in excess SMRT molecules. The excess amount of SMRT will interact with ER and likely interrupt the binding of E2. Therefore, this model provides a simple scenario for the cross talk between the RA and E2. The ultimate goal of this project is to test this hypothesis and to explore the possibility of using SMRT mutants as a mean to intervene breast cancer cell proliferation. We believe that these studies should contribute to understanding the molecular basis of hormone action and breast cancers.

Our objectives:

We aim to understand the molecular basis through which RA interacts with E2 in regulating proliferation of HBC cells. We will test the proposed hypothesis. Through understanding molecular mechanisms, we hope to provide new insights into development of therapeutic strategies that will lead to a better treatment of breast cancer. Note that the third objective in the original proposal is deleted because of the reduction of funding periods according to referee's comments. Because several other groups have been working on similar project with the N-CoR proteins, our study will focus on SMRT in order make competitive progress. The following are the specific objectives for this proposal.

1). To analyze the expression and regulation of SMRT genes in different HBC cells. The status of SMRT gene expression will be compared to that of RAR and ER. We will also investigate the regulation of SMRT expression after treatment with RA and E2.

2). To investigate physical interactions between SMRT and the steroid hormone receptors including ER, PR, and GR upon treatment with hormones or anti-hormones. These studies should establish a role of SMRT in cross talk between retinoids and steroids.

Together, these studies will establish a link between retinoids and steroid hormone action, as well as the role of nuclear receptor corepressors in breast cancers.

(6) Body

Methods

1. To analyze the expression and regulation of SMRT in breast cancer cells

Several HBC cell lines will be obtained and established in the P.I.'s laboratory. These include five ER-positive cells (T-47D, MCF-7M, MDA-MB-361, BT474 and MDA-MB-134) and six ER-negative ones (MDA-MB-231, MDA-MB-330, BT 20, Hs0578T, MDA-MB-453, HBL100). To test whether any of these cell lines express the co-repressors, RNA will be isolated from these cells. Equal amounts of RNAs will be analyzed by Northern blot technique to determine the expression of SMRT. Through hybridization with ³²P-nucleotide labeled probes, we will determine the mRNA levels of SMRT by comparison with the actin message. Furthermore, we will determine the message levels of ER, PR and RAR α in the same set of experiments. By these studies, we expect to determine the expression levels of corepressors and nuclear receptors. A correlation between the expression level of RAR α and the ER message in breast cancer cells has been reported (23). It is thus conceivable that the corepressors may also express at a higher level in the ER-positive cells. These results should establish a correlation between the expression level of corepressors and nuclear receptors in the breast cancer cells. Next, we will investigate whether retinoic acid or steroids could regulate the expression of SMRT. It has been reported that estrogen and progesterone can up regulate the expression of RAR α gene (22, 24, 28), while RA down-regulates the expression of ER (25). Thus, hormones might regulate the expression of corepressors as well. To study this possibility, we will treat the breast cancer cells with different hormones and analyze their effects on the expression levels of SMRT by Northern blot. It is known that the sizes of SMRT mRNA are quite large (about 7 to 9 kb) and are relatively rare in most cells (5, 15). If no clear signal can be detected in total RNA, we will isolate poly A(+) RNA for the Northern blot analysis or analyze the messages by RNase Protection Assay using commercially available reagents (e.g. Ambion Inc.).

From these analyses, the status of SMRT gene expression will be determined in different breast cancer cells and compared to the expression levels of RAR, ER and PR. If the expression levels of SMRT are abnormally high in these cells, it may suggest that transcriptional repression of certain target genes through the SMRT pathway is involved in the oncogenesis process of HBC. Furthermore, we anticipate a correlation between the expression levels of RAR and SMRT

in ER-positive breast cancers. These results should indicate the involvement of co-repressors in the RA-mediated growth arrest of breast cancer cells. On the other hand, it is also possible that the expression levels of SMRT in these breast cancer cells do not have significant differences from other cells. In which case it may suggest that other genes such as the RAR α itself is directly involved in the growth arrest of breast cancer cells upon RA treatment. These experiments should also reveal the possibility of transcriptional regulation of SMRT by hormones. In this case, we will continue to isolate the 5'-genomic DNA fragment for SMRT and the effects of hormone on the promoter activities of these isolated genomic DNA will be test. These studies should provide essential information about the possible involvement and the transcriptional regulation of nuclear receptor corepressors in the development of breast cancers.

2. To investigate the involvement of nuclear receptor corepressors in the cross-talks between retinoids and steroids

The mechanisms underlying the cross-talks between signal transduction pathways for RA and E2 are unknown, but are keys to understanding the fundamental basis of hormone-induced growth arrest of HBC cells. One possible explanation for this phenomenon is the existence of molecular integrators between these two pathways. It is possible that SMRT is such an integrator. The interaction between SMRT and ER raises an interesting possibility that ER may interact with co-repressors in a stable manner upon the binding to certain hormone analogs. To reveal these possibilities, we will first test whether SMRT interacts with other steroid hormone receptors, including progesterone receptor (PR), glucocorticoid receptor (GR), and androgen receptor (AR). The GST-SMRT fusion proteins will be expressed and purified, and will be used for GST-pull down assay to detect protein-protein interactions with ER, PR, GR, and AR. These steroid hormone receptors will be synthesized and radiolabeled in vitro using the in vitro translation system (TNT system, Promega, Inc.). We will test the effects of hormones and anti-hormones on these protein-protein interactions. The receptor complexes will be analyzed by SDS-PAGE followed by autoradiography detection of the labeled receptors. From these studies, we expect to answer whether SMRT could interact with the steroid receptors upon binding to certain hormone analogs, such as antihormone that could induce distinct conformational changes in the receptor (1). These studies should also answer whether the co-repressors may play a role underlying the inability of anti-hormone in activating the receptors. In addition to the GST-pull down assay, we will also conduct far-western analysis to confirm the protein-protein interactions. Furthermore, we will perform the two-hybrid interaction tests using both yeast and mammalian cells, as well as immunoprecipitation using nuclear extracts isolated from hormone-treated cells. These experiments will further confirm the biological significance of these protein-protein interactions. Our laboratory is specialized in these techniques and these experiments should generate fruitful results.

Results

In this report, I will discuss the progress of each task and will specify deviations that we have made to overcome unforeseen difficulty.

Task 1: Month 1-4: Collecting the human breast cancer cell lines and isolation of total cellular RNA.

We have obtained and established several breast cancer cell lines in our laboratory. These include several ER-positive cells (T-47D, MCF-7M, MDA-MB-361, BT474 and MDA-MB-134) and ER-negative cells (MDA-MB-231, MDA-MB-330, BT 20, Hs0578T, MDA-MB-453). The normal breast epithelial cell line HBL100 is also established, which will be used as a control. These cells are currently maintained in Dulbecco's modified Eagle's medium (DMEM) and have been expanded in the laboratory. Long term stocks are preserved under liquid N₂ for later experiments.

We have isolated total RNA from these cells using the RNazol kit (Test Inc.). Aliquots of these RNAs are stored in a -70°C freezer for later experimental needs. We measured the RNA concentration by spectrophotometer and found that these RNA samples have an OD₂₆₀/OD₂₈₀ ratio between 1.8 to 2.2, indicating that these RNAs are of significant purity. Furthermore, we have analyzed these RNA samples by using formaldehyde-agarose gels followed by ethidium bromide staining. The results show two sharp 28S and 18S ribosomal RNA bands, indicating the integrity of these RNA samples. Therefore, these RNAs are suitable for analysis of expression of specific gene.

Task 2: Month 2-7: Northern blot analysis of the expression levels of SMRT in different breast cancer cells.

We have conducted Northern blot analysis for the expression of SMRT in these cancer cells. Using the random priming kit (Boehringer), we generated a DNA probe containing part of the human SMRT cDNA. The probe was hybridized to the RNA blot overnight in Express hybridization buffer (Clontech) according to manufacturer's recommendation. Preliminary results indicate that SMRT expresses as a 9-kb band at relatively low level in these breast cancer cells. We found that the signal of SMRT on the Northern blot was weak. It normally takes upto 3 days exposure in order to see a band on the Northern blot. Therefore it is difficult to determine the relative expression levels of SMRT in these breast cancer cells using the Northern blot technique with total RNAs.

To tackle this problem, we have determined to try two different approaches. First, we will use RNase protection assay to quantitate SMRT message using the isolated total RNA. An RNase protection kit will be purchased from Ambion Inc. Second, we have tried to measure the protein level of SMRT by Western blot using an affinity purified anti-SMRT polyclonal antibody. To do so, we raised both rabbit and chicken polyclonal antibodies from a commercial source against different regions of the SMRT protein, including the C-terminal, the middle domain and the most N-terminal region. So far, we have completed the production of one antibody. The antibody was affinity purified using an antigen coupled cyanogen bromide-sepharose affinity column. We have determined the specificity of this antibody by Western blot. The Western blot was developed using the ECL plus kit purchased from Amersham Inc. Work on characterizing the other antibodies is currently in progress.

By using the antibody, we detected the expression of SMRT in all human breast cancer cells. Interestingly, we found that the level of SMRT in HBL100 cells is relatively lower than in other cell types (see figure 2 in Appendices). Work is currently in progress to confirm this finding by using other antibody.

Task 3: Month 8-12: Treatment of breast cancer cells with various RAR and ER hormones, isolation of RNA after treatment, and northern blot analysis of the expression levels of SMRT after treatment of the cells with different compounds.

Because total RNA Northern blot for SMRT message did not work well. We are currently redoing the analysis using Western blot and RNase protection assay. First, we test the expression level of SMRT protein after treatment of different cells with either RA or E2. So far, we have not observed any major change after such treatment. These results suggest that SMRT level may not be regulated by hormones. Therefore, it is possible that the involvement of SMRT in the cross talk between RA and E2 is at the level of protein-protein interactions before and after binding of SMRT to different nuclear receptors. Work is currently in progress to determine how SMRT interacts with RAR and ER after hormone and anti-hormone treatments.

We have found that SMRT interacts with RAR very strongly in vitro and vivo (Figure 3 in Appendices). Such interaction is sensitive to RA, suggesting a conformational change of the receptor upon ligand binding. Such conformational change is responsible for the dissociation of SMRT from RAR. Using similar assay, we also detected the interaction between ER and SMRT. Interestingly, such association is slightly enhanced by E2 treatment. We have tested the effect of anti-estrogen Tomoxifen on the interaction. We found that tomoxifen also promote the association between SMRT and ER, but only slightly (not shown).

Related to the proposed work, we have also characterized the receptor interaction and transcriptional repression domains of SMRT. The following are some of our findings, which have been reported in the journal *Molecular Endocrinology* (34).

Two receptor domains are essential for interaction with SMRT

Deletion mutants in the carboxyl and amino termini of TR and RAR were used to analyze the contribution of different regions in the receptors for protein-protein interaction with SMRT. Figure 1A shows the domain structure of TR and the relative position of individual helices in the LBD as determined by X-ray crystallography. The sequence at the C-terminus region around helix 11 and 12 is also shown for both TR and RAR. ³⁵S-methionine labeled TR or RAR deletion mutants were hybridized to GST-SMRT and GST-RXR in far-Western analyses in the absence of hormone (Fig. 4B). The relative strengths of these interactions are summarized in Fig. 4C.

Full length TR (1-456) associates well with both SMRT and RXR, and the interaction with SMRT can be drastically reduced upon hormone treatment. A residual weak interaction was consistently observed in the presence of ligand, consistent with previous observations. Carboxyl-terminal truncation at residue 441, which deletes helix 12, results in a mutant that interacts

normally with RXR but that exhibits enhanced interaction with SMRT. Further truncation at residue 423, which removes part of helix 11, reduces the interaction with SMRT back to a level similar to that of wild type TR. In contrast, this deletion markedly reduces interaction with RXR. Further deletions that remove additional helices (helices 8,9,10) result in barely detectable interaction with SMRT, and no interaction with RXR. These results suggest that the helix 12 inhibits SMRT association while the helix 11 may promote the association.

Amino-terminal truncation of TR at residue 173, which removes the DBD, does not affect the interaction with either SMRT or RXR. Further N-terminal deletion to residue 260, which removes the first and second helices of the TR LBD, markedly impairs SMRT association. No interaction with RXR by this mutant was detectable. Similarly, C-terminal deletion of helix 12 from RAR (1-403) also increases interaction with SMRT as compared with that of wild type RAR (1-462). Further deletion to residue 395, which removes part of helix 11, diminishes the enhanced interaction to a level comparable with that of full length RAR, and ligand has little effect on the interaction. Together, these results identify two distinct interacting domains at the N-terminal hinge and C-terminal helix 11 regions of the receptor LBD that may act synergistically to promote interaction with SMRT. We find that the other two RAR isoforms, β and γ , also interact with SMRT in a ligand-reversible manner, although the interactions observed are weaker compared to that with RAR α (Fig. 4D). The interactions of both RAR β and RAR γ with RXR were not affected by ligand treatment.

Two SMRT domains mediate differential interactions with nuclear receptors

The finding that two regions of TR are essential for protein-protein interaction with SMRT suggests that SMRT might also contain duplicated receptor interacting domains. Several deletion mutants of SMRT were utilized to test this possibility in a far-Western blot, and the results are summarized in figure 5A. The GST-fusions of these SMRT mutants were overexpressed, and the purified proteins (Fig. 5B, lanes 1, 2) or crude extracts (lanes 3,4,5) were analyzed for interaction with ^{35}S -RAR and TR. We found that SMRT(981-1495 Δ) interacts equally well with both RAR and TR in the absence of ligands. RAR, but not TR, also interacts with degradation products of SMRT(981-1495 Δ). Similarly, several fast migrating products of SMRT(1086-1291) also interact well with RAR, but not with TR (lanes 4). These results indicate that RAR and TR may interact differently with SMRT. Consistent with this speculation, we find that SMRT(982-1291) (lanes 2) as well as SMRT(1086-1291) interact more strongly with RAR than with TR. In contrast, the C-terminal fragment (1260-1495 Δ) interacts better with TR than RAR (lanes 5). All these interactions were found sensitive to hormone treatment (Fig. 5B and data not shown). Together, these results identify two independent receptor interacting domains (RID-1 and RID-2) of SMRT that appear to display different affinities to TR and RAR.

Two SMRT repression domains

In addition to the receptor-interacting domains, SMRT and N-CoR also contain strong transcriptional repression activity at their N-terminal regions. To define the minimal region needed for repression, serial SMRT deletion mutants were generated, and their repression activities were analyzed using transient transfection (Fig. 6A). Consistent with previous

observations, full length as well as N-SMRT (aa 1-981) repress basal transcription strongly and in a dose-dependent fashion (rows 2, 3), while C-SMRT (aa 982-1495Δ) exhibits minimal repression (row 4) compared to Gal4 DBD alone (row 1). Further deletion from the C-terminus of N-SMRT reveals that amino acids 743 to 981 are not necessary for repression (row 5), while deletion to residue 475 reduces the repression effect about two-fold (row 6). These results suggest that amino acids 475 to 981 may contribute in part to SMRT repression. Further C-terminal deletion to residue 337 drastically interferes with repression (row 7), indicating that the N-terminal boundary of this SMRT repression domain-1 (SRD-1) is located between amino acids 337 and 475. Truncation from the N-terminus reveals that amino acids 1 to 134 are dispensable for repression by SRD-1 (row 8). Further deletion to residue 337 abolishes repression (row 9). These results indicate that the C-terminal boundary of the SRD-1 is within amino acids 134 to 337. When the SMRT fragment between amino acids 475 and 981 was tested for repression, we found that this fragment also strongly repressed basal transcription (row 10). Together with the observation that amino acids 743 to 981 are not important for repression, these results may define amino acids 475 to 743 as a second, independent SMRT repression domain (SRD-2).

Sequence comparison between SMRT and N-CoR reveals that they share about 45% identity within both SRD-1 and SRD-2, suggesting potential functional conservation. Therefore, we tested whether the two SRD corresponding regions of N-CoR also contain repression activities. Consistent with a previous observation, amino acids 1-312 and 752-1016 exhibit strong repression activities (Fig. 6B, rows 2 and 3). The two N-CoR domains corresponding to SRD-1 and SRD-2 also yield 10- to 30-fold repression (rows 4 and 5), similar to the repression effects observed by SRD-1 and SRD-2. These two additional N-CoR repression domains are termed N-CoR repression domain 3 and 4 (NRD-3 and NRD-4) and the two N-terminal repression domains NRD-1 and NRD-2. Together, these results indicate that both SMRT and N-CoR contain multiple, independent transcriptional repression domains.

It is possible that lack of repression in some of these SMRT/N-CoR deletion mutants is due to lack of protein expression. Thus, we analyzed expression of these constructs by both in vitro translation and Western blot analysis after transient transfection. We find that all constructs used in this experiment express approximately equal amount of Gal4 DBD fusion proteins in vitro (Fig. 6C) and that the repression defective mutants express well in vivo (Fig. 6D). The low levels of signal in Gal4 DBD and SMRT(337-475) are perhaps due to fewer methionines in these two polypeptides compared to others, since Western blot analysis of these constructs detects strong signals (Fig. 6D). These results indicate that lack of repression by certain SMRT/N-CoR deletion mutants are not due to lack of protein expression.

Task 4: Month 13-15: Isolation of SMRT genomic clones and identification of potential hormone response elements. By this time, we expect to answer whether the expression of SMRT is regulated by hormones.

Because the effect of hormone on the expression level of SMRT is not dramatic (only less than two two-fold difference), we think that it might not yield significant finding on studying the regulation of SMRT gene expression at this point. We will isolate the mouse genomic clone of the SMRT coding region, instead of the regulatory region. We think that the genomic clone

will be useful in the future for understanding the organization of SMRT gene and for generation of null-SMRT mouse. This work is currently in progress, and will continue in the second year of this project.

Objectives 2: To investigate the involvement of nuclear receptor corepressors in the cross-talks between retinoids and steroid hormones.

Task 5: Month 16-19: Evaluating the protein-protein interaction between nuclear receptor corepressors SMRT and steroid hormone receptors ER, PR, GR and AR in vitro.

We have conducted several experiments on analyzing the interaction between SMRT and ER. We found that SMRT can interact with ER in either the absence or presence of hormone. Work are still in progress to investigate the interaction between SMRT and other steroid receptor and the significance of such interaction.

Task 6: Month 19-21: Investigating the effects of hormone and anti-hormone binding on the protein-protein interaction between steroid hormone receptors and corepressors SMRT.

This task is currently in progress and will be completed in the second year.

Task 7: Month 20-24: Analyzing the protein complex of receptors and corepressors in human breast cancer cells. By this time, we should be able to answer whether the co-repressor may play an integrator role between the retinoids and steroid hormone actions in breast cancer cells.

This task is currently in progress and will be completed in the second year.

(7) Conclusions

In summary, this project has progressed well. We will continue our current study to understand the role of nuclear receptor corepressor in breast cancer. So far, we have completed the analysis of SMRT gene expression in human breast cancer cells. Although more work are needed in order to make a conclusion, the results have demonstrated a possible role of SMRT in the development of breast cancers. We found that in the cancerous cells, SMRT level is elevated compared to the normal breast epithelial cells. In addition, we have further characterized the transcriptional repression domain of SMRT, revealing two independent repression domains at the N-terminal region of SMRT. More interestingly, we have also identified another isoform of SMRT, which is similar in size to the nuclear receptor corepressor N-CoR. Study on this novel isoform of SMRT is currently in progress. In the next year, we will include our study on the role of this SMRT isoform in breast cancers. Therefore, we have generated several preliminary observations, which provides an important basis for the study in the subsequent year.

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(9) Appendices

Figure 1. Model of SMRT action in breast cancer.

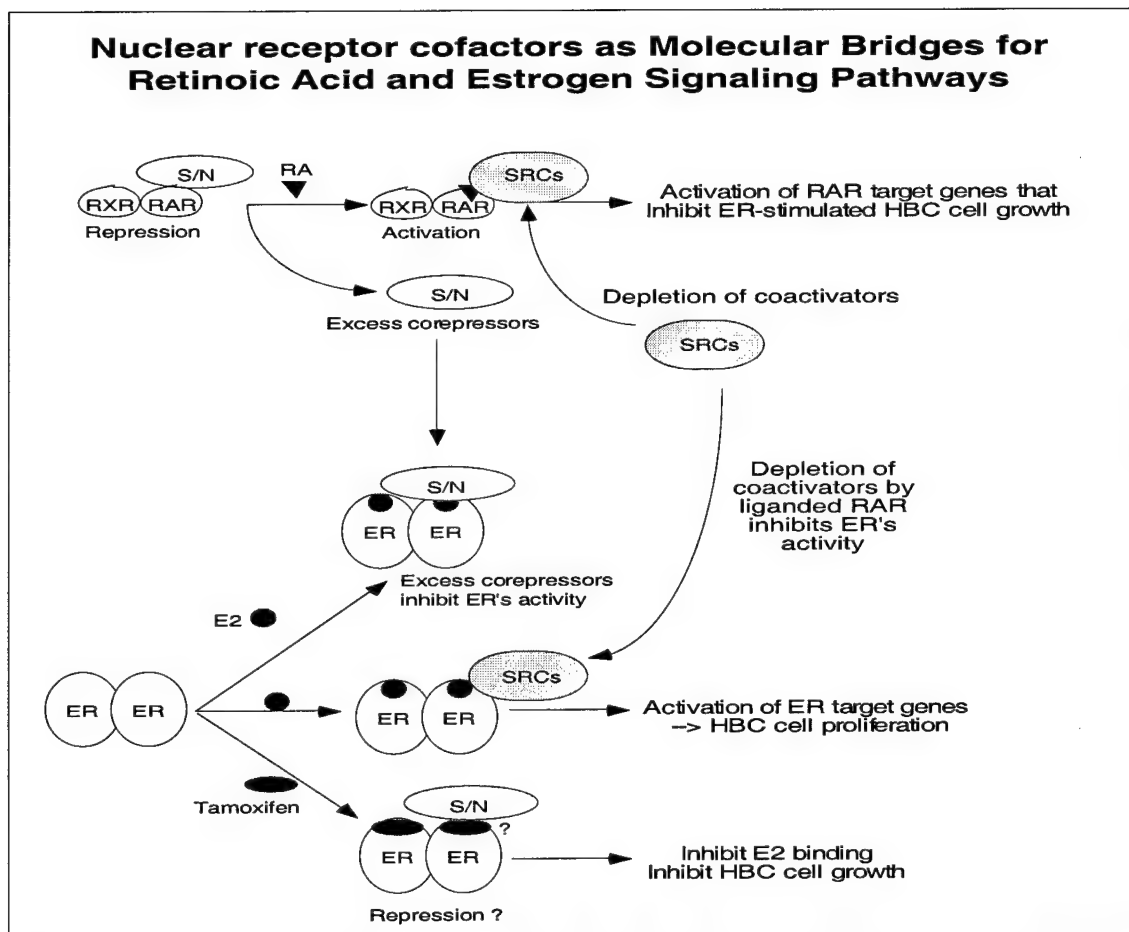


Figure 2. SMRT expression in breast cancer cells. Total cell extracts were separated on a SDS-PAGE, blotted onto a nitrocellulose filter and hybridized with an affinity purified anti-SMRT antibody on total cell

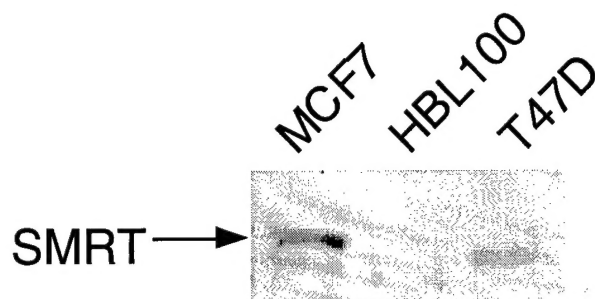


Figure 3. SMRT interacts with ER. We blotted the purified GST-C-SMRT on nitrocellulose filters and hybridized with ^{35}S -methionine labeled nuclear receptors in the absence or presence of hormones. We used *all-trans* RA for RAR, 9-*cis* RA for RXR, T3 for TR and 17 β -estradiol for ER at 1 μM concentration). The last lane shows the GST-SMRT fusion protein on the gel after staining by commassie blue. These results indicate that SMRT are not only capable of interacting with unliganded RAR and TR, but also capable of interacting with ER.

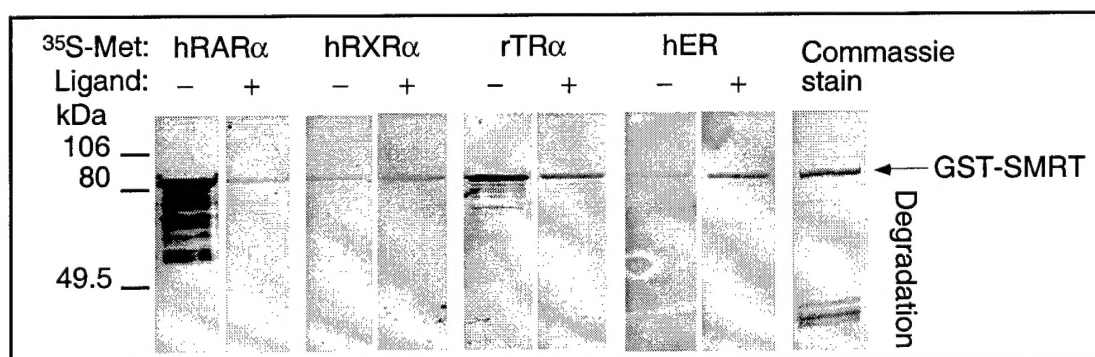


Figure 4. Two nuclear receptor domains interact with SMRT. (A) Domain structure of hTR β . (B) Protein-protein interactions between receptors and SMRT or RXR in far-Western analyses. (C) Summary of relative levels of interactions between receptor mutants and SMRT or RXR. (D) Human RAR β and RAR γ interact with SMRT in a ligand-reversible manner in far-Western blots.

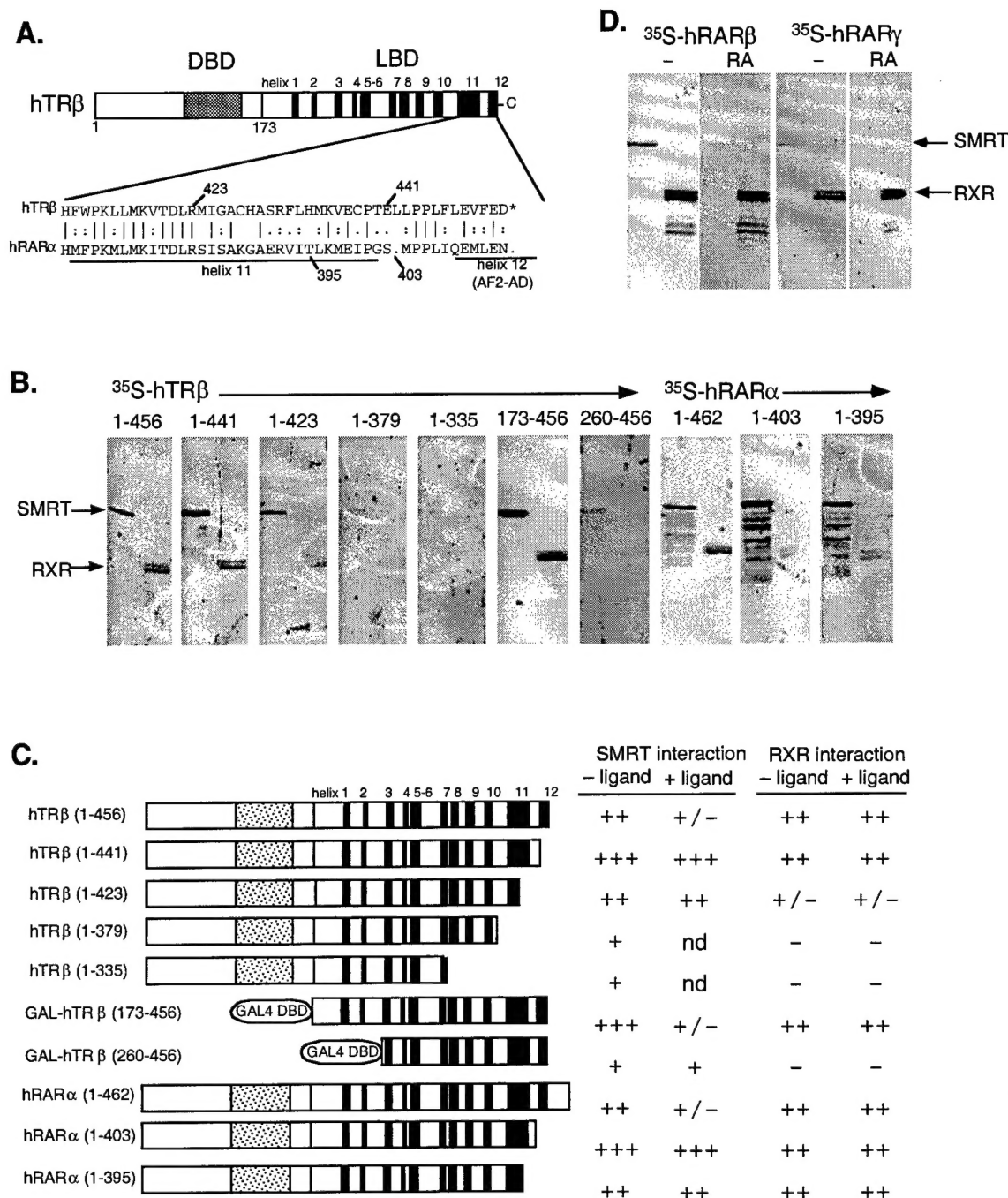





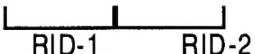


Figure 5. Two SMRT domains interact with the receptors. (A) Summary of SMRT deletion mutants used in this experiment and their relative levels of interaction with nuclear receptors in far-Western analyses shown in (B). The amino acids encoded by the SMRT mutants are shown in parentheses. Bound-RAR and TR were detected by autoradiography, and the relative levels of interaction were scored from background level (-) to strong (+++). The column numbers in each panel correspond to constructs shown in (A). Partially purified GST fusion proteins were used in lanes 1 and 2 and total cell extracts in lanes 3, 4, and 5. RID, receptor interacting domain. + T3, plus 1 μ M T3 (triiodothyronine), Q, glutamine-rich domain; H, putative helical region; Δ , an internal deletion between amino acids 1330 and 1375 resulting from alternative splicing.

A.

		RAR	TR	TR(+T3)
1. GST-SMRT (982-149 Δ)		+++	+++	+/-
2. GST-SMRT (982-1291)		+++	+	-
3. GST-SMRT (982-1086)		-	-	-
4. GST-SMRT (1086-1291)		+++	++	-
5. GST-SMRT (1260-149 Δ)		+	++	+/-
				

B.

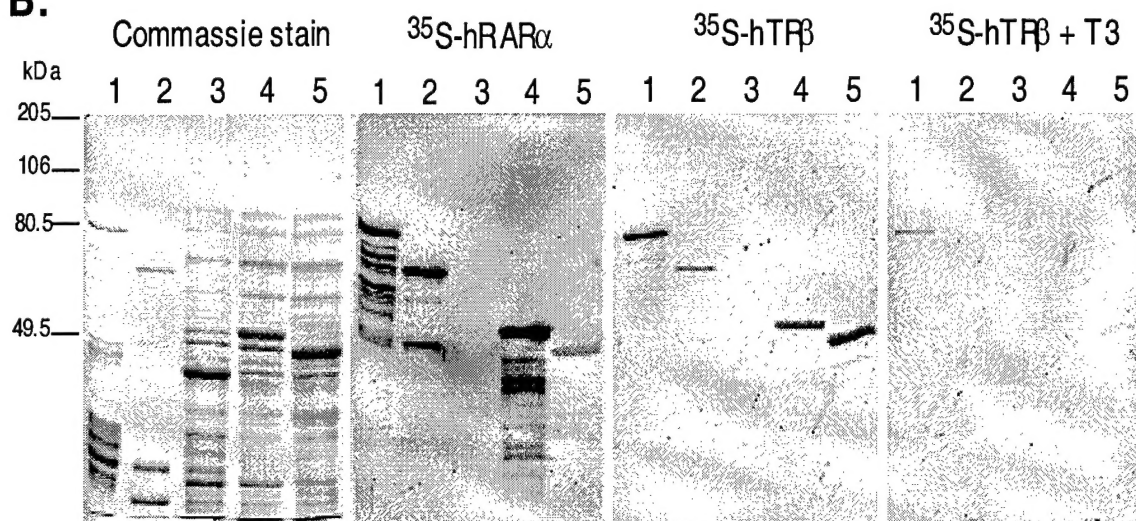


Figure 6. Multiple transcriptional repression domains. (A) Deletion mapping of the repression domains of SMRT. The transcriptional repression activities were analyzed by transient transfection in CV-1 cells. (B) Deletion mapping of the N-CoR repression domains (NRDs). The N-CoR domains are aligned with those of SMRT in (A).

